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RESEARCH

Agrobacterium-Mediated Transformation and Insertional Mutagenesis in *Colletotrichum acutatum* for Investigating Varied Pathogenicity Lifestyles

Pedro Talhinhas · S. Muthumeenakshi · João Neves-Martins · Helena Oliveira · S. Sreenivasaprasad

Abstract *Colletotrichum acutatum* is a cosmopolitan pathogen causing economically important diseases known as anthracnose on a wide range of hosts. This fungus exhibits varied pathogenicity lifestyles and the tools essential to understand the molecular mechanisms are still being developed. The transformation methods currently available for this species for gene discovery and functional analysis involve protoplast transformation and are laborious and inefficient. We have developed a protocol for efficient *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *C. acutatum*. Using this protocol we were able to transform *C. acutatum* isolates belonging to different genetic groups and originating from different hosts. The transformation efficiency was up to 156 transformants per 10^4 conidia, with >70% transformants showing single location/single copy integration of T-DNA. Binary vector pBHt2-GFP was constructed, enabling green fluorescence protein tagging of *C. acutatum* strains, which will be a useful tool for epidemiology and histopathology studies. The ATMT protocol developed was used to identify putative pathogenicity mutants, suggesting the applicability of this technique for rapid generation of a large panel of insertional mutants of *C. acutatum* leading to the identification of the genes associated with the varied lifestyles.

Keywords *Colletotrichum acutatum* · *Agrobacterium tumefaciens*-mediated transformation · Hygromycin B resistance · Green fluorescent protein expression · Insertional mutagenesis · Pathogenicity mutants

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Introduction

Colletotrichum acutatum has a worldwide distribution, causing diseases generally known as anthracnose on a wide range of hosts including woody and herbaceous crops, ornamentals, fruits and conifers. *C. acutatum* displays high levels of genotypic and phenotypic diversity and worldwide populations cluster into at least eight distinct molecular groups. *C. acutatum* populations show pathogenic variability and cross-infection potential in relation to a number of hosts. The pathogen has varied infection strategies and complex epidemiology, exhibiting pathogenic and non-pathogenic lifestyles on target hosts, non-target crops and weeds [1, 2]. In view of the economic importance of the diseases caused by this pathogen on crops such as almond, citrus, olive and strawberry, in-depth studies on epidemiology and infection strategies have been carried out [3]. However, very little information is available on the molecular mechanisms regulating varied pathogenicity life styles and host preference and the basic tools required are only beginning to be developed by various groups. Horowitz and co-workers [4] transformed germinating conidia of a strawberry pathogenic isolate of *C. acutatum* by electroporation and selection on hygromycin, but only a maximum of six transformants could be obtained from 10^7 conidia with 5 μg of transforming DNA. Chung and co-workers [5] used the sulphonylurea (chlorimuron ethyl) selection system for the transformation of *C. acutatum* protoplasts with an acetolactate synthase gene (*sur*) cassette from *Magnaporthe grisea*. The transformation frequencies varied from 0 to 17.9 per μg DNA using

10^7 protoplasts. In both these investigations neither REMI nor the use of homologous sequences increased the transformation efficiency [4, 5].

Agrobacterium tumefaciens-mediated transformation (ATMT) is a powerful tool for large-scale random mutagenesis, and efficiently targeted gene disruption, based on the transfer of the T-DNA into the recipient host genome (e.g. [6–8]). This technique has been shown to be applicable to yeasts [9] as well as filamentous fungi [10]. More recently, the potential of ATMT as a genetic analysis tool in functionally diverse fungi has been demonstrated [11–13] for green fluorescence protein (GFP) expression [14, 15], homologous gene replacement [16–18] and tagged mutagenesis [19, 20].

In this article, we describe an ATMT protocol for the efficient transformation of *C. acutatum* and GFP expression, overcoming the difficulties and low efficiencies previously reported [4, 5]. We also characterised the type of T-DNA integrations in the fungal genome, and tested the potential application of this method for insertional mutagenesis to generate pathogenicity mutants to aid in future gene discovery studies.

Materials and Methods

Fungal Isolates, Bacterial Strains and Culture Conditions

Colletotrichum acutatum isolate CA397, a strawberry pathogen belonging to group A2 [1, 21], was grown on Potato Dextrose Agar (PDA, Sigma-Aldrich, UK) at 25°C. Isolates PT29 (from white lupin), PT108, PT169 and PT227 (from olive) and CR46 (from grapevine) belonging to *C. acutatum* [1, 22, 23] used in transformation experiments were grown under similar conditions. Sensitivity of *C. acutatum* to hygromycin and sulphonylurea [5] was tested in plate assays using isolate 397. PDA plates containing varying concentrations of hygromycin dissolved in water (0, 50, 150, 250 and 375 μg/ml) and sulphonylurea dissolved in dimethylformamide (0, 10, 30, 50 and 75 μg/ml) were inoculated centrally with a 3 mm inoculum plug and the radial growth of the colony was measured after 8 days. Conidial suspensions were prepared from 5-day PDA cultures by adding ca. 5 ml sterile distilled water to the plates, releasing spores and mycelium with a sterile loop and filtering through a sterile 200 μm glass filter. Number of conidia in the suspension was counted using a haemocytometer and adjusted to required concentrations. *Agrobacterium tumefaciens* strain AGL1 (A. Soares) was grown on LB agar plates supplemented with 20 μg/ml rifampicin (Sigma-Aldrich) and 75 μg/ml carbenicillin (Sigma-Aldrich) at 26°C for 2 days. AGL1 containing a binary vector was grown on LB agar plates supplemented with 20 μg/ml rifampicin and 50 μg/ml kanamycin (Sigma-Aldrich) at 26°C for 2 days. Binary vector pBHt2 [24], based on pCAMBIA 1300 (AF234296; Cambia, Australia) containing the hygromycin B resistance (*hph*) gene under the *Aspergillus nidulans trpC* promoter (S. Kang) was transformed into *Escherichia coli* TOP 10 cells (TOPO TA cloning kit, Invitrogen, UK). The new cell line was grown on LB medium supplemented with 50 μg/ml kanamycin at 37°C. Plasmid pPgpd/GFP010 [25], containing the *gfp* cassette with the *A. nidulans gpdA* promoter and *trpC* terminator (A. Soares) was transformed into TOP 10 cells. The new cell line was grown on LB medium supplemented with 100 μg/ml ampicillin at 37°C.

Binary Vector Construction and Transformation into *Agrobacterium tumefaciens*

A 2366-bp *Sac*I and *Xba*I (Roche Diagnostics, Germany) digested fragment of pPgpd/GFP010 containing the *gfp* cassette was isolated by gel purification using the QIAquick gel extraction kit (Qiagen, UK). *Sac*I and *Xba*I digested and dephosphorylated (shrimp alkaline phosphatase; Roche Diagnostics) pBHt2 was prepared using the QIAquick PCR Purification kit. Digested pBHt2 and the pPgpd/GFP010 fragment were ligated using T4 DNA ligase (Roche Diagnostics). The 2366-bp fragment was cloned into the *Sac*I and *Xba*I sites within the multiple cloning site region of pBHt2, in opposite direction to that of the *hph* gene cassette to construct pBHt2-GFP (Fig. 1). pBHt2-GFP was transformed into TOP 10 *E. coli* cells. Correct orientation of pBHt2-GFP was confirmed by restriction mapping with *Hin*dIII, *Sac*I and *Xba*I and also by PCR verification of the *gfp* and *hph* (described in "*PCR Confirmation of hph or gfp Gene in the Transformants*"). All plasmid isolations were performed using the Plasmid Mini Kit (Qiagen).

For preparing the *A. tumefaciens* competent cells, AGL1 strain was grown in liquid LB to an OD_{660} of 0.15. Cells were harvested by centrifugation (5000 rpm 10 min 4°C), washed thrice in 10 ml chilled 100 mM HEPES and once in 10% glycerol by centrifugation 5000 rpm 10 min 4°C between each wash. The cells were resuspended in 1 ml 10% glycerol for electroporation (alternatively, for chemically competent cells, resuspension was in 1 ml of 20 mM $CaCl_2$). Competent cells (40 μL) were mixed with 200 ng plasmid (either pBHt2 or pBHt2-GFP) and added to a pre-chilled electroporation cuvette. Electroporation was carried out by applying 2.5 kV (at 400 Ω) with capacitance set at 25 μF (Gene Pulser and Pulse Controller, Biorad, UK). Alternatively, 100 μl of chemically competent cells were mixed with 1 μg plasmid DNA, frozen in liquid nitrogen

Co-cultivation medium	Binary vector	No. of conidia per membrane	Mean no. of transformants per membrane
IMAS	pBHt2	10^3	0.29
		5×10^3	29.63
		10^4	156.29
		5×10^4	Countless
		10^5	
	pBHt2-GFP	10^3	0.00
		5×10^3	17.85
		10^4	44.88
		5×10^4	Countless
		10^5	
IM	pBHt2	10^3	0.44
		5×10^3	60.19
		10^4	129.71
		5×10^4	Countless
		10^5	
	pBHt2-GFP	10^3	2.24
		5×10^3	32.13
		10^4	81.90
		5×10^4	Countless
		10^5	

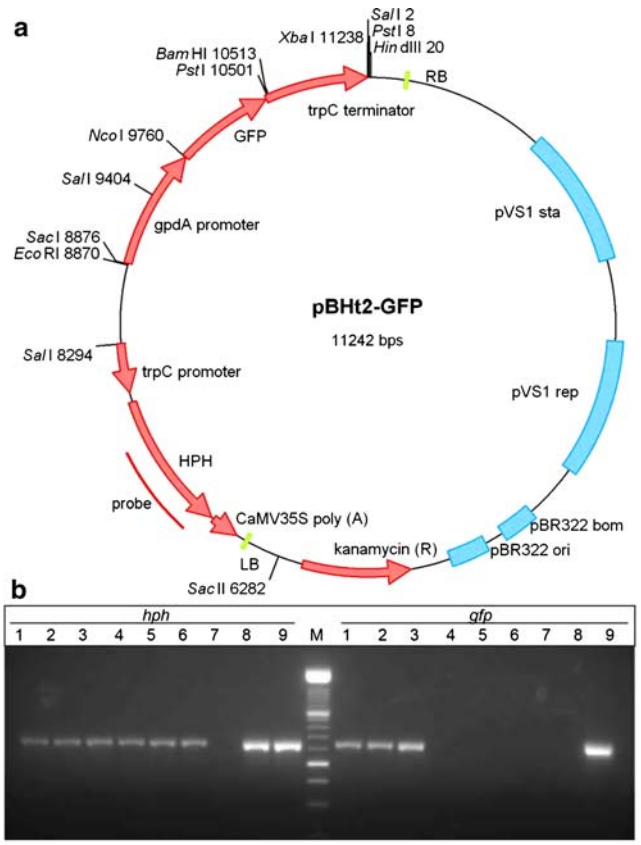


Fig. 1 Left (table)—Number of *Colletotrichum acutatum* transformants obtained (discrete colonies growing on the selection medium and confirmed by subsequent subculturing; average of six transformation experiments) in relation to the number of conidia, the binary vector used (pBHt2 or pBHt2-GFP) and the presence (IMAS) or absence (IM) of acetosyringone (200 μM) in the co-cultivation medium (countless—coalescent colonies/continuous mycelial mat). Right (**a**)—Map of binary vector pBHt2-GFP, obtained by cloning the green fluorescence protein gene, *gfp* (including *A. nidulans gpdA* promoter and *trpC* terminator; [25]), into the *Sac*I/*Xba*I sites within the multiple cloning site region of pBHt2 [26]; T-DNA, including the *gfp* and *hph* (hygromycin B resistance) genes defined by the left border (LB) and the right border (RB); probe (as used for Southern analysis) region marked. Right (**b**)—Fragments amplified by PCR using the *hph* (left panel) or *gfp* (right panel) specific primers with DNA from six *C. acutatum* transformants obtained with AGL1 containing binary vectors pBHt2-GFP (lines 1–3) or pBHt2 (lines 4–6); M—marker XIV (Roche Diagnostics; bright bands are 2642 bp, 1000 bp and 500 bp); Lane 7: CA397—wild type (negative control); Lane 8: pBHt2—containing *hph* gene (positive control for PCR with *hph*-specific primers) but not *gfp* (negative control for PCR with *gfp*-specific primers); Lane 9: pBHt2-GFP—containing both *hph* and *gfp* (positive control for both PCRs)

for 5 min and incubated at 37°C for 35 min. After transformation, cells were immediately recovered by adding 1 ml LB and incubated for 3 h at 26°C 150 rpm. Cells were plated in LB agar plates supplemented with 20 μg/ml rifampicin and 50 μg/ml kanamycin at 26°C for 2 days. Selected colonies were subcultured and the new cell lines were stored at −80°C.

Agrobacterium tumefaciens-Mediated Transformation and GFP Expression

AGL1 cell line containing either pBHt2 or pBHt2-GFP recovered from −80°C storage was first grown on LB plates containing 30 μg/ml kanamycin and 20 μg/ml rifampicin at 26°C for 2 days. A single colony was transferred to LB medium amended with 30 μg/ml kanamycin and grown overnight at 26°C. Subsequently, 2 ml of the cells were used to inoculate a 25 ml culture grown overnight at 26°C and 150 rpm in minimal medium (MM). The composition of MM was 11.8 mM K_2HPO_4 + 10.7 mM KH_2PO_4, pH 7.0; 2.0 mM $MgSO_4$. $7H_2O$; 2.6 mM NaCl; 456.4 μM $CaCl_2.2H_2O$; 10.1 mM glucose; 9 μM $FeSO_4.7H_2O$ and 3.8 mM $(NH_4)_2SO_4$ (adapted from [27, 28]). Optical density (OD_{660}) of the cell suspension was recorded at 660 nm and an appropriate volume was used to prepare 50 ml of the suspension at 0.15 OD_{660} in the induction medium (IM). The composition of IM was same as MM, with the following modifications: 40 mM 2-(N-Morpholino)-ethanesulphonic acid, pH 5.3 and 0.5% glycerol (adapted from [9, 28]). Cells from 20 ml of the suspension in IM were pelleted by centrifugation for 8 min

at 2500 rpm and resuspended in 50 ml IM containing 30 μg/ml kanamycin and 200 μM acetosyringone (3′,5′ Dimethoxy-4′-hydroxyacetophenone, Acros, Belgium). A freshly prepared stock of acetosyringone (100 mM in ethanol) was used. Cells were incubated for 6 h at 26°C and 150 rpm. Petri plates (9 cm diam.) containing IM or IMAS (IM amended with 200 μM acetosyringone) with 2% agar and overlaid with a cellophane membrane were used to spread a *C. acutatum* isolate conidial suspension (100 μl) together with an identical volume of *A. tumefaciens* cell suspension. Different concentrations of the conidial suspension were tested (ranging from 10^3 to 10^6 conidia per plate), while the volume of bacterial suspension was kept constant [29]. Co-cultivation plates were routinely incubated for 48 h at 22°C, although 20 and 25°C were also tested. The membranes were then removed, cut into six to eight pieces and placed on a selective medium of PDA amended with 250 μg/ml hygromycin (Invivogen, USA) and 400 μg/ml cefotaxime (Duchefa, Netherlands). Fungal colonies growing on the selective medium were transferred to $PDA_{hyg,cf}$ or $YEG_{hyg,cf}$ (a liquid medium containing 56 mM glucose and 0.2% yeast extract, amended with the same antibiotics). Transformants were maintained on PDA_{hyg}. Stability of a random selection of transformants was tested by subculturing at least thrice on PDA without the selective antibiotic and then checking their capacity to grow on PDA_{hyg}. A *C. acutatum* conidial sample not co-cultured with *A. tumefaciens* cells but handled as described above throughout the selection procedure served as negative control.

GFP expression in the transformants obtained with AGL1-pBHt2-GFP was assessed by epifluorescence microscopy. Actively growing hyphae from PDA_{hyg} or YEG_{hyg} cultures were observed under ultraviolet light (350–400 nm) on an Olympus IX70 microscope at 400× magnification. Wildtype isolate was used as control.

Isolation of Genomic DNA from Putative Transformants

To extract DNA for PCR assays, transformants were grown on PDA_{hyg} or YEG_{hyg} liquid without agitation. A small amount of mycelium (approx. 2-mm diameter clump) was resuspended in 1 ml 1 mM Tris–HCl containing 100 μm glass beads and 3% (w/v) Chelex 100 (Sigma-Aldrich) and vortexed for 1 min. The suspension was subjected to three freeze-boil cycles of 1 min each in liquid nitrogen and boiling water, with a final 5 min boiling. This was followed by vortexing for 1 min and incubation for 40 min at 55°C. The mix was centrifuged 5 min at 13000 rpm and the top 100 μl recovered and stored at -20°C. To extract DNA for Southern analysis, transformants were grown in 30 ml of YEG_{hyg} at 25°C for 3–5 days. Mycelium was filtered through sterile filter paper in a Buchner funnel, frozen in liquid nitrogen, freeze-dried and grounded to a fine powder. DNA extraction was performed using 10 mg of mycelial powder using GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) following manufacturer's instructions. DNA obtained in 200 μl elution buffer was further concentrated by ethanol precipitation and resuspended in 20 μl water.

PCR Confirmation of *hph* or *gfp* Gene in the Transformants

Primers HPHF—5′ cgatcttagccagacgagcg 3′ and HPHR—5′ ttgccctcggacgagtgctg 3′ derived from GenBank sequence (L76273) were used to amplify a 643 bp *hph* fragment. Primers eGFP361—5′ ggccacaagttcagcgtgtc 3′ and eGFP998—5′ agctcgtccatgccgagagt 3′ [30] were used to amplify a 638 bp *gfp* fragment. PCR was performed with 5 μl DNA, 1 μM each primer (final concentration) and REDTaq ReadyMix PCR Mix (Sigma-Aldrich) in a final volume of 25 μl on a Proteus II (Helena Biosciences, UK) thermalcycler. PCR programme included one cycle of 5 min at 94°C, 30 cycles of 45 s at 94°C, 30 s at 55°C (for both *hph* and *gfp*) and 1 min at 72°C and a final cycle of 5 min at 72°C.

Southern Analysis of T-DNA Integration in the Transformants

DNA samples (1 μg) from a random selection of transformants were digested with either *Bgl*II (Roche Diagnostics) or *Hind*III (Invitrogen), overnight at 37°C using 10 U of the enzyme and the appropriate buffer in a 20 μl reaction. Restriction fragments were separated by agarose (0.8%) gel electrophoresis overnight (ca. 20 h) at 0.67 V/cm, along with DIG-labelled marker II (Roche Diagnostics). The 643 bp fragment of the *hph* gene (200 pg) loaded on the gel 2 h before the end of electrophoresis served as positive hybridisation control. DNA was transferred to positively charged nylon membrane (Roche Diagnostics) following the standard Southern blotting protocol [31]. After blotting, the membrane was washed in 6 × SSC for 2 min and the DNA was fixed by incubation at 80°C for 1 h and UV cross-linking at 0.35 J/cm^2. The blots were rinsed in hybridisation solution (5 × SSC + 0.1% v/v Sarkosyl + 0.02% SDS) and then twice incubated for 1 h at 65°C in pre-warmed pre-hybridisation solution (hybridisation solution + 2% w/v casein blocking agent) in a rotating cylinder (Techne Hybridiser, UK). The 643 bp *hph* gene fragment was labelled with digoxigenin using PCR DIG Probe synthesis kit (Roche Diagnostics),

gel checked and purified and was used as a probe. Salmon sperm DNA (200 µl of 10 mg/ml stock; Sigma-Aldrich) was added to 200 ng of the DIG-labelled *hph* probe. The probe mixture was denatured and mixed with a minimal volume of pre-warmed pre-hybridisation solution (10 ml for a 20 × 20 cm membrane) and added to the membrane and incubated overnight at 65°C. The membrane was washed twice with 3 × SSC + 0.1% SDS (first wash at room temperature for 5 min and second at 65°C for 15 min), twice with 1 × SSC + 0.1% SDS for 15 min at 65°C and twice with 0.2 × SSC + 0.1% SDS for 15 min at 65°C. For chemiluminescent detection, the membrane was first washed at room temperature on a platform shaker with 100 mM Tris HCl pH 7.5 + 150 mM NaCl for 5 min, then with 100 mM Tris HCl pH 7.5 + 150 mM NaCl + 2% casein blocking agent for 30 min and finally with an identical solution containing Anti-Digoxigenin-AP Fab Fragments (1:5000 dilution; Roche Diagnostics) for 30 min. Following this, the membrane was washed twice with 100 mM Tris HCl pH 7.5 + 150 mM NaCl to remove any unbound antibody conjugate, once with 100 mM Tris HCl pH 9.5 + 100 mM NaCl + 50 mM $MgCl_2$ and finally with 20 ml of previous solution containing 200 µl CDP-star (Roche Diagnostics) for the detection of nucleic acids. Membrane wrapped in cling film was used for autoradiography with 2–10 min exposure times.

Pathogenicity Assay of Insertional Mutants

Transformants were grown on PDA_{hyg} and inoculated on green 'Golden Delicious' apples which were previously surface-disinfected with NaClO 0.05% and rinsed in distilled water. The fruits were inoculated by making an approximately 0.8 cm-deep hole using a cork borer and placing a 0.5 cm diameter plug of actively growing culture of the transformants. Fruits inoculated with the wildtype isolate CA397, and isolate AC58, originally obtained from apples with anthracnose symptoms and also an uncultured PDA plug, served as controls. Fruits were incubated for 13 days at 25°C under 100% relative humidity. The reactions were recorded as lesion type and diameter (mm), production of mycelia and sporulation. Based on these data, an overall disease severity score (ranging from 0 to 10) was assigned. In a first screen, each transformant was inoculated once, with six inoculation spots per apple. In the second screen, each of the transformants exhibiting distinctive differences in pathogenicity plus appropriate controls were inoculated in four replicates, at four inoculation spots per apple, in a totally randomised design. For pathogenicity bioassays with strawberry, immature fruits (green-white stage) of 'Camarosa' variety were used. Wild type isolate CA397 and insertional mutants 1417 and 1577 were each inoculated on to 10 strawberries. Surface-disinfected fruits were inoculated with 10 µl of a conidial suspension (10^6 spores/ml) and incubated under the conditions described above.

Results and Discussion

Co-cultivation of *C. acutatum* conidia with *A. tumefaciens* strain AGL1 containing a binary vector pBHt2 or pBHt2-GFP consistently yielded high frequency of transformants in more than five independent experiments. *A. tumefaciens* strain AGL1 was used in this study, as it has been found to be more efficient than strains such as EHA105 and LBA1126 in ATMT of both asco- and basidio-mycete fungal species [32, 33]. *C. acutatum* isolates from citrus were considered moderately tolerant to selection agents such as hygromycin, bialaphos, benomyl and geneticin/G418 [5]. In our tests, *C. acutatum* isolate 397 also showed tolerance to hygromycin at concentrations used routinely with other fungal species, and only at 250 µg/ml and above the growth was completely inhibited (Fig. 2). Tests with sulphonylurea also revealed that *C. acutatum* isolate 397 had high tolerance to this agent up to 75 µg/ml (data not shown). Consequently, in this study, hygromycin was used at a higher concentration (250 µg/ml) than that commonly used (100 µg/ml) with other fungi (e.g. [20, 34]). Similarly, in a recent study reporting a high-throughput ATMT system for insertional mutagenesis in *Magnaporthe oryzae*, 350 µg/ml of hygromycin was used [35]. After 48 h co-cultivation of *C. acutatum* conidia and AGL1 cells containing a binary vector and prior to transfer to the selection medium, a slight mycelial mat was visible covering the cellophane disc, corresponding to 48 h fungal growth on control plates. However, upon transfer of the discs to selection medium containing hygromycin at 250 µg/ml, discrete colonies of transformants started appearing after 4 days. At lower hygromycin concentrations tested, it was

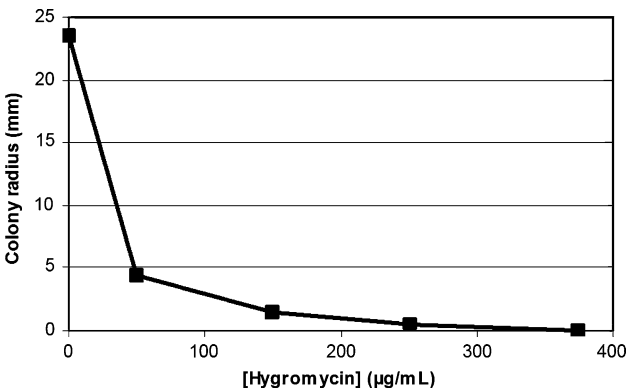


Fig. 2 Growth curve showing the sensitivity of *Colletotrichum acutatum* isolate 397 to various concentrations of hygromycin

difficult to distinguish transformed colonies from the background growth (data not shown).

In various ascomycete fungi, 10^6 conidia have been used in ATMT experiments (e.g. [20, 24]). However, with *C. acutatum*, at this conidial concentration, the number of putative transformants growing in selection plates was too high and it was difficult to isolate discrete colonies (data not shown). Therefore, an experiment was carried out to test various parameters such as the concentration of the conidia, binary vector and the presence or absence of acetosyringone in the co-cultivation medium in order to develop an efficient protocol to reliably isolate individual transformants of *C. acutatum*. Results clearly showed that 10^4 conidia per co-cultivation plate were optimal with an average transformation efficiency of between 45 and 156 transformants (Fig. 1). Even at 5×10^4 conidia per co-cultivation plate, a large number of putative transformants appeared leading to coalescent colonies or a mycelial mat. Acetosyringone is a compound released by wounds in *A. tumefaciens* plant hosts in nature, which the bacterium uses as a signal for infection and T-DNA transfer [24] and its addition has been reported to increase the efficiency of ATMT [12]. However, with *C. acutatum* no substantial difference in the transformation efficiency was observed between the presence (IMAS) and absence (IM) of acetosyringone in the co-cultivation medium (Fig. 1). This suggests that acetosyringone added to the bacterial induction medium, prior to co-cultivation, was sufficient for the ATMT process. Co-cultivation of *C. acutatum* conidia and *A. tumefaciens* cells was routinely carried out at 22°C. Experiments conducted at 20 and 25°C, showed some degree of increased transformation efficiency at 25°C (results not shown). Further, pre-germination of conidia is frequently used prior to co-cultivation in fungal ATMT to increase the chances of infection and transformation of developing hyphal cells (e.g., [20]). In *C. acutatum*, pre-germination of conidia is strictly required for direct transformation by electroporation [4]). However, in our ATMT experiments, incubation of conidia on co-cultivation plates for 24 or 48 h prior to the addition of bacterial cells did not improve the efficiency of transformation of *C. acutatum*. This in fact, decreased the efficiency of generating discrete colonies of transformants, presumably due to growth enhancement by pre-germination (data not shown). Interestingly, similar difficulties were observed with another *Colletotrichum* species, *C. graminicola* when the co-cultivation period was extended to 5 days [29]. Further, the *A. tumefaciens* strain AGL1 appears to be very efficient with various *Colletotrichum* spp. such as *C. acutatum* tested in this study, *C. gloeosporioides* (P. Ramos, pers. comm.) and *C. graminicola* [29].

In view of the polyphagus nature of *C. acutatum* and the existence of at least eight different phylogenetic groups namely A1–A8 [1], besides the transformation of isolate CA397, experiments were conducted to test the wider applicability of the method. Other *C. acutatum* isolates tested include PT29 (group A1 from white lupin), PT108, PT169 and PT227 (representing groups A2, A4 and A5, respectively, associated with olive anthracnose) and CR46 (group A3 from grapevine), which have been genetically characterised previously [1, 22, 23]. ATMT experiments with *C. acutatum* isolates PT29, PT108, PT169, PT227 and CR46 using AGL1 containing the binary vector pBHt2 yielded $\sim$120 and $\sim$150 transformants from 10^4 conidia without and with acetosyringone in the induction medium, respectively. At least 25 transformants tested for each isolate showed stable hygromycin resistance. These results demonstrated that the ATMT technique developed might be used as an efficient tool for any *C. acutatum* isolate. All ATMT experiments were performed with appropriate controls. It is worth noting that, in the controls, after the co-cultivation period and transfer of the cellophane disc to selection plates only a diffuse background growth from the germinated spores was visible, but no clear colonies were formed. However, in areas where the cellophane disc was not in direct contact with the selection medium, such as ends of the membrane that were in contact with the side wall of the Petridish or over air bubbles that lifted the membrane above the selection medium, further mycelial growth was observable. This emphasises the importance of properly laying down the co-cultivation discs ensuring full contact with the selection medium including at the edges. Some of these colonies appearing in the control plate with the wild type isolate only grew slightly on PDA containing 250 μg/ml hygromycin, but not in YEG liquid medium containing an identical concentration of hygromycin, making this a better choice for effective selection of transformants. We routinely transfer putative transformants to sterile 1.5 ml eppendorf tubes containing 1 ml YEG_{hyg} before further subculture as necessary. This is a very useful method for avoiding any false positives as well as enabling the efficient management of hundreds of transformants, saving space as well as safe handling of each transformant avoiding any cross-contamination. The transformation frequency of 45–156 transformants per co-cultivation plate with 10^4 conidia achieved in this work is clearly more efficient compared to previous reports of six transformants from 10^7 conidia with 5 μg DNA [4] and 0–17.9 per μg DNA using 10^7 protoplasts [5]. Moreover, the protocol developed is also applicable to a wide range *C. acutatum* isolates from different host and genetic groups.

A collection of 20 randomly selected transformants (10 each from either IM or IMAS co-cultivation medium) was tested for mitotic stability of the transgene integration. This was carried out by three serial subcultures in non-selective medium, followed by transfer back to selection medium,

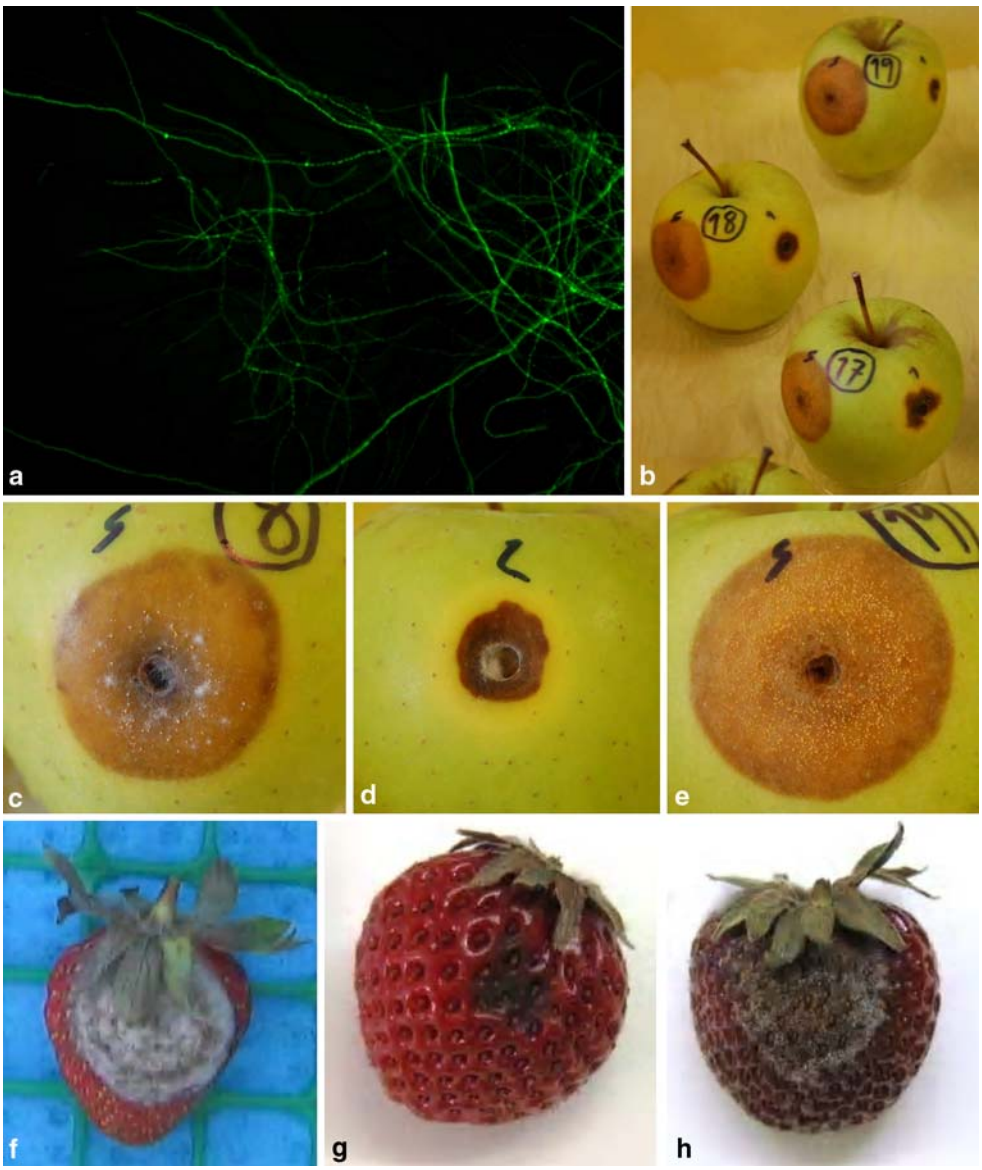
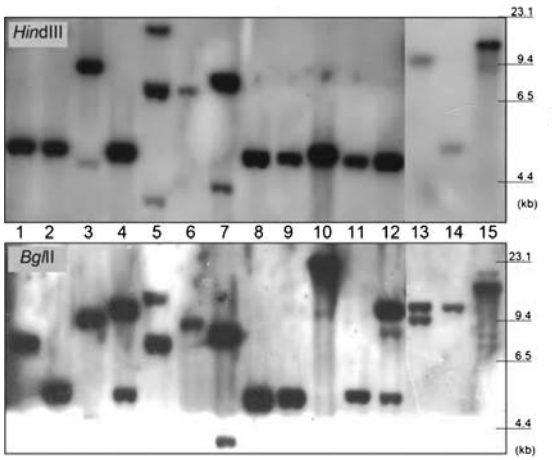


Fig. 3 (**a**) Hyphae of a *Colletotrichum acutatum* transformant generated with vector pBHt2-GFP expressing the green fluorescent protein visualised by epifluorescence microscopy (400×). (**b–h**) Pathogenicity bioassay of *C. acutatum* insertional mutants generated by *Agrobacterium tumefaciens*-mediated transformation, showing disease reactions obtained after 13-days incubation at 25°C under 100% relative humidity along with the wild type isolate CA397 (positive control). (**b**) Bioassay with apple fruits serves as a high-throughput screen, as 3–5 transformants can be tested on each fruit without interference from each other. (**c–e**) Symptoms obtained with the wild type (CA397), a low-pathogenicity mutant (1417) and a high-pathogenicity mutant (1577), respectively, in screening bioassays on apple; (**f–h**) Symptoms obtained with the wild type (CA397), mutant 1417 and mutant 1577, respectively, on strawberry fruits

where all 20 transformants grew in the presence of hygromycin. Further, epifluorescence microscopy of a selection of transformants obtained with binary vector pBHt2-GFP clearly showed *C. acutatum* hyphae expressing the GFP (Fig. 3). Hyphae of the wild type isolate or transformants produced with pBHt2 did not show any green fluorescence under the same conditions. GFP-tagged *C. acutatum* strains are likely to facilitate future epidemiological and host colonisation studies.

A simple and quick DNA extraction method and a PCR assay were tested to verify the presence of transgenes in putative transformants, prior to Southern analysis or pathogenicity phenotype screening. Putative transformants were screened by PCR using specific primers for *hph* and *gfp*. Figure 1 shows an example of two sets of three transformants generated with vectors pBHt2 and pBHt2-GFP, accompanied by the appropriate controls. Transformants obtained with vector pBHt2-GFP yielded *hph* and



Number of bands				
Restriction enzyme		Number of transformants	Type of integration and frequency
*Bgl*II	*Hin*dIII		
1	1	26	Single site (72.2%)
1	2	1	Single site, repeat (2.8%)
2	1	2	Two sites (16.6%)
2	2	4	
3	1	1	Three sites (5.6%)
3	3	1	
4	2	1	Four sites (2.8%)

Fig. 4 Left (figure)—Southern analysis of T-DNA integration events in *Colletotrichum acutatum* transformants generated by *Agrobacterium tumefaciens*-mediated transformation; blots containing genomic DNA digested either with *Hin*dIII (single site within the T-DNA; top panel) or *Bgl*II (no site within the T-DNA; bottom panel) were hybridised with a digoxigenin-labelled fragment of the hygromycin resistance gene, *hph* probe. Right (table)—Type and frequency of T-DNA integration events in *C. acutatum* transformants based on the Southern analysis

gfp fragments of the expected sizes. Similarly, transformants obtained with pBHt2 yielded *hph* fragments but not *gfp* fragment. This rapid protocol provides a high-throughput PCR assay for the confirmation of >100 putative transformants per day.

Southern analysis of a selection of transformants using an *hph* gene probe was carried out to characterise the T-DNA integration events in ATMT of *C. acutatum*. This was aimed at analysing single or multiple integrations as well as single copy or multi-copy integration of T-DNA at a location. For this experiment, transformants were chosen to represent the different variables tested, namely the type of binary vector (pBHt2 or pBHt2-GFP) and the presence (IMAS) or absence (IM) of acetosyringone in the co-cultivation medium. Genomic DNA from 36 transformants was digested with either *Bgl*II (no restriction site in T-DNA) or *Hin*dIII (one restriction site in T-DNA outside of the probe region) and hybridised with the *hph* probe (Fig. 1). Transformants with single copy integration at a location should yield a single band with both the restriction enzymes and those with multiple integrations are expected to yield identical number of bands with the two enzymes. A multi-copy (repeat) integration in a single location will yield a single band with *Bgl*II, expected to be at least twice the size of T-DNA (>10 kb for pBHt2-GFP and >5.3 kb for pBHt2) and two bands with *Hin*dIII, one of which, representing the monomer, would be the same size as the T-DNA. Complex integrations such as both single copy and repeats in different locations in the same transformant would be more difficult to interpret, although this would be expected to result in more than one *Bgl*II band of different sizes and a 'strong' *Hin*dIII band (corresponding to multiple monomers from the repeat) plus some 'weak' *Hin*dIII bands.

Southern analysis results based on the number and size of bands obtained with the two enzymes for each transformant revealed that single copy integration was most common (lanes 1, 2, 6, 8–11 and 14 in Fig. 4), representing 72.2% of the transformants analysed (Fig. 4). Up to four copies of T-DNA were detected in some examples representing 25% multiple integrations (lanes 3–5, 7, 12, 13 and 15 in Fig. 4). A single case of multi-copy (T-DNA repeat) integration was detected (data not shown), with two *Hin*dIII bands and one *Bgl*II band. In this transformant, the *Bgl*II band was ca. 25 kb indicating the integration of a five-copy repeat of the T-DNA from vector pBHt2-GFP. The frequency of single copy integration obtained with ATMT of *C. acutatum* in this study is comparable to those reported with other *Colletotrichum* spp. such as *C. trifolii* [36] and *C. lagenarium* [19] and clearly higher than in *C. graminicola* [29]. Further, among the 36 transformants analysed, 27 were from the IMAS co-cultivation medium containing acetosyringone and nine were from IM without the inducer. Interestingly, the proportion of single copy integrations was similar (ca. 67%) among these transformants, although acetosyringone has been reported to both increase [37] and decrease [38] the number of T-DNA integrations in different systems. Nearly 33% of the transformants yielded *Bgl*II bands of a similar size (ca. 5.5 kb), along with larger bands in some cases (e.g. lanes 2, 4, 8, 9, 11 and in Fig. 4). Considering that almost all of the 36 transformants analysed were from independent transformation experiments, the results suggests the possibility of integration hotspots in the *C. acutatum* genome. However, despite this, 66.7% of the transformants tested showed unique *Bgl*II profiles. Based on this and the overall 72.2% single integration events, an estimated 48.2% of the

C. acutatum transformants should contain a single copy random T-DNA integration in their genome.

Varied pathogenicity lifestyles of *C. acutatum* on different hosts as well as on different organs and cultivars of the same host are well recognised [2, 3, 39]. Discovery of pathogenicity genes in this key pathogen will enable further understanding of mechanisms driving the varied lifestyles. T-DNA-tagged mutants altered in pathogenicity offer an attractive approach for the identification of the genes. To analyse the pathogenicity phenotypes in a collection of nearly 1500 *C. acutatum* transformants, an apple-based bioassay was used. Apples were preferred over strawberries, the original host of the wildtype isolate, because of easier availability throughout the year as well as the suitability to test multiple (six to eight) transformants on a single fruit and also due to less susceptibility to saprophytic invasion. The apple bioassay enabled the testing of 211 transformants in a single experiment. Most of the transformants caused extensive lesion or rot around the inoculation site comparable to the controls (Fig. 3). However, several transformants showed an altered pathogenicity phenotype including five that produced a very small lesion or no lesion and 16 were noted for enhanced severity (Figs. 3 and 5). The pathogen was re-isolated from some of these lesions on selective medium and their identity [23] as well as the presence of the *hph* transgene was confirmed by PCR assays. These 21 transformants were reinoculated on apples in a second, fully replicated and randomly designed experiment and at least two were confirmed as low-pathogenicity mutants with no sporulation, slight or no mycelial formation and none to a very small lesion. Two other transformants were confirmed as high-pathogenicity mutants with abundant spore masses on the fruit surface, medium to high mycelial formation and a very large lesion area (data not shown). In pathogenicity bioassays on strawberries, insertional mutant 1417 showed markedly reduced level of infection with small areas of necrosis (Fig. 3g), compared to the wild type (Fig. 3f). This particular mutant showed a very similar phenotype in the initial screening using the apple bioassays (Fig. 3d). Mutant 1577 revealed heavy colonisation of the strawberry fruit and large area of necrosis with spore masses erupting (Fig. 3h), as with the apple bioassay (Fig. 3e). These results clearly demonstrate the value of the ATMT methodologies developed and the pathogenicity bioassays described for understanding the molecular mechanisms regulating varied infection processes in *C. acutatum*.

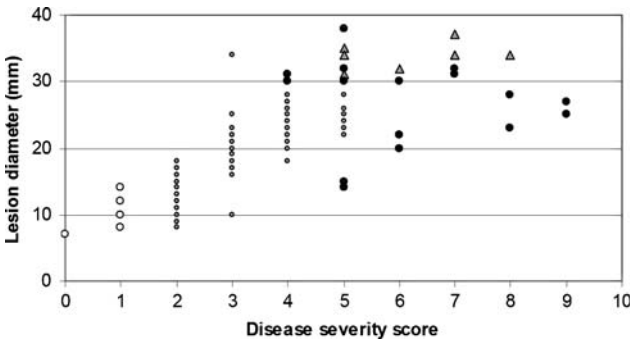


Fig. 5 Assessment of the pathogenicity phenotype of *Colletotrichum acutatum* transformants obtained by *Agrobacterium tumefaciens*-mediated transformation, following inoculation on apples (incubated for 13 days at 25°C under 100% relative humidity). Distribution pattern of the disease severity score (0, no lesion; 10, large lesion with sporulation) and lesion diameter (mm) for the 211 transformants tested; five transformants displaying a lower level of infection (white circles) and 16 displaying a higher level of infection (black circles) compared to the controls (wild-type isolate CA397 and apple isolate AC58 shown in grey triangles) were selected for further screening; grey circles represent other transformants that were not tested further

Conclusions

This is the first report of successful ATMT of *C. acutatum*, which is a wide host range cosmopolitan pathogen. The protocol described is clearly more efficient compared to the transformation methods reported for *C. acutatum* and offers various advantages. The transformation frequency of 45–156 per 10^4 conidia is significantly higher than the 0–18 transformants per μg DNA reported for this species [4, 5]. Moreover, ATMT enables the transformation of conidia, instead of protoplasts, preparation of which is expensive and laborious, and also the lytic enzymes that often have batch variations. Efficiency of the ATMT of *C. acutatum* is comparable to that of various ascomycetes including *C. graminicola* [29] and higher than other *Colletotrichum* spp. such as *C. trifolii* [36], *C. gloeosporioides* [10], *C. lagenarium* [19] and *C. higginsianum* [40] where only one to three transformants per 10^4 conidia were reported. We have also described a protocol that enables high-throughput PCR analysis of the *C. acutatum* transformants. Further, we have demonstrated that ATMT of *C. acutatum* leads to high levels (>70%) of single integration events and this protocol can be used to rapidly generate a panel of T-DNA tagged pathogenicity mutants. PCR-based assays to recover the flanking sequences from T-DNA insertional mutants as well as T-DNA-based targeted gene knockout methods have recently been reported with various fungi (e.g. [18]). Thus, the ATMT of *C. acutatum* is a useful tool for gene discovery and gene function analysis to gain novel insights into the varied pathogenicity lifestyles exhibited by this economically important pathogen.

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